1-1-2017

Molecular characterization of the 5' and 3' ends of the dengue virus genome and its usefulness in pathogenesis studies

Alicia Alejandra Rosales Munar
Universidad de La Salle, Bogotá

MOLECULAR CHARACTERIZATION OF THE 5' AND 3' ENDS OF THE DENGUE VIRUS GENOME AND ITS USEFULNESS IN PATHOGENESIS STUDIES

Alicia Alejandra Rosales Munar

Universidad de La Salle
Department of Basic Sciences
Bogotá D.C.
2017

MOLECULAR CHARACTERIZATION OF THE 5' AND 3' ENDS OF THE DENGUE VIRUS GENOME AND ITS USEFULNESS IN PATHOGENESIS STUDIES

Alicia Alejandra Rosales Munar

Research work submitted as a partial fulfillment of the requirements for the degree of:

Biologist

Director:

José Aldemar Usme Ciro. *BSc., MSc., PhD*

Codirector:

Óscar Javier Ramos Real, *MSc, PhD*

Universidad de La Salle
Department of Basic Sciences
Bogotá D.C.
2017

TABLE OF CONTENTS

ABBREVIATIONS

DENV	Dengue virus
DENV-1	Dengue virus type 1
DENV-2	Dengue virus type 2
DENV-3	Dengue virus type 3
DENV-4	Dengue virus type 4
ml	Milliliters
µl	Microliters
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
UTR	Untranslated regions

1. Abstract

Dengue is one of the mosquito-borne diseases of major importance in public health, which has spread worldwide, especially in tropical and subtropical regions, as a result of globalization, precarious urbanization, and expansion of the mosquito vector. Although it has been extensively studied at the molecular level, most of the dengue virus (DENV) genomes at public databases lack or have uncorroborated ends of the untranslated regions (UTRs), becoming a limitation when conducting studies of association between the genetic background of the virus and the clinical outcome of the disease. In an attempt to solve this problem, the present study aims to characterize the 5' and 3' ends of dengue virus, to provide complete information for future studies of viral pathogenesis. The 5´ UTRs of twenty-seven Colombian DENV isolates (from patients with or without warning signs or severe disease) were readily amplified through a standard RACE approach while the 3´ UTRs required the development of a modified RACE protocol, For the cDNA synthesis of the 5' UTR, a specific anti-sense primer for each serotype was used, which were located at the coding region of the C or prM/M proteins. The cDNAs were purified and subsequently polyadenylated using the terminal transferase. By using an oligodT and an internal reverse primer located upstream of the first primer used for cDNA synthesis, the 5' UTRs were successfully PCR amplified and sequenced. For the molecular characterization of the 3' UTR, an adenine tail was directly added to the positive-sense viral RNA using the *E coli* poly (A) polymerase, which allowed the hybridization of the oligo-dT for the synthesis of cDNA, subsequent PCR amplification and Sanger sequencing. The results allow to demonstrate the successful characterization of the whole dengue virus UTRs and to assess the potential impact of point mutations at the RNA secondary structure level.

Keywords: Genome ends, sequencing, dengue virus, RACE, *E. coli* Poly (A) polymerase.

2. Introduction

Dengue is considered to be one of the most important arthropod borne pathogens, since the number of infected individuals has increased in the last two decades (Brathwaite Dick *et al.*, 2012). It has been estimated that 50 to 100 million infections occur worldwide each year; In addition, infections occur in approximately 100 countries in the Asian-Pacific region, Americas, Middle East and Africa (Brathwaite Dick *et al.*, 2012; WHO, 2009). Dengue may manifest in the host in two ways; Asymptomatic and symptomatic, the former may be presented as DF (Dengue Fever), Dengue Hemorrhagic Fever (DHF) or Dengue Shock Syndrome (DSS), or dengue without or with warming signs and severe dengue, according to the more recent WHO classification (WHO, 2009). Currently, other atypical manifestations involving the damage of body organs or systems known as "severe forms of dengue with organ compromise" and frequently associated with extreme severity and death are recognized (Salgado, 2008).

Dengue virus (DENV) is transmitted through the bite of infected female mosquitoes belonging to the genus *Aedes*, mainly *Aedes aegypti*, which is distributed in tropical and subtropical countries, where about 3.6 billion people live (Rodriguez-Roche & Gould, 2013). The life cycle of the DENV begins when the mosquito feeds on the blood of an infected person, generating the infection of the mosquito midgut epithelial cells; then, the viral particles generated in these cells are released to invade other organs such as the salivary glands; The infection of the human host occurs when the mosquito injects its saliva-containing DENV particles during the blood meal (Velandia & Castellanos, 2011).

DENV is a serocomplex of four globally distributed and closely related species (serotypes DENV-1, -2, -3 and -4) belonging to the family *Flaviviridae*, genus *Flavivirus* (Lindenbach *et al.*, 2013; (Twiddy *et al.*, 2002). Its genome consists of a positive-sense single-stranded RNA of about 11 Kb, which encodes for three structural (capsid, pre-membrane and envelope) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (Guzman *et al.*, 2010). At the 5' and 3' untranslated regions (5’ and 3’ UTR, respectively), the DENV genome contains several evolutionarily conserved structural elements involved in viral protein synthesis, genome replication and pathogenesis (Bäck & Lundkvist, 2013; Chiu, Kinney, & Dreher, 2005; Hussain & Asgari, 2014; Yu, Nomaguchi, Padmanabhan, & Markoff, 2008).

The 5' UTR has a length of 95 to 101 nucleotides and contains two structural domains, the stem-loop A (SLA) and the stem-loop B (SLB) (Selisko, Wang, Harris, & Canard, 2014), in addition to the 5' UAR (5' upstream AUG region) element that is complimentary to the 3' UAR during genome circularization. Two regions of the SLA domain interact with the viral RNA-dependent RNA polymerase (RdRp) promoting viral RNA synthesis. The SLB domain contains a 16-nucleotide-long (5' UAR) that together with the 5' cyclization sequence (5' CS) is involved in genome cyclization, an essential process for viral replication (Filomatori, Iglesias, Villordo, Alvarez, & Gamarnik, 2011; Friebe & Harris, 2010).

Moreover, the 3' UTR comprises a stretch between 384 and 462 nucleotides and lacks of a poly (A) tail. It is composed of three domains (I, II and III). The domain I, also known as variable region (VR) extends about 50 to 120 nucleotides and is located just after the polyprotein stop codon (Zhou *et al.*, 2006). It was recently reported that this region contains a viral miRNA with a role in DENV autoregulation in C6/36 cells by targeting the coding sequence of the viral NS1 protein (Hussain & Asgari, 2014). The domain II includes a DB (Dumbbell) structure with a double tandem repeat containing the conserved sequences CS2 and RCS2 (repeated CS2), these conserved sequences were proposed to be involved in viral translation and RNA synthesis (Gebhard, Filomatori, & Gamarnik, 2011). Finally, the domain III is the most conserved region of the DENV 3' UTR and contains two elements. The first one is the 3' conserved cyclization sequence (3' CS), a short element of 11 nucleotides complementary to the 5' CS located at the capsid-coding sequence (Filomatori *et al.*, 2011). The second one is the 3' stem-loop structure (3' SL) with approximately 79 nucleotides in length (Paranjape & Harris, 2010). This element contains the 3' UAR sequence which is complementary to the 5' UAR at the 5' UTR. Both elements (3' CS and 3' SL) promote the genome circularization through base pairing with its complimentary 5' sequence enabling the binding DENV and host proteins implicated in genome replication and protein translation.

Although the genome of DENV has been extensively studied at the molecular level, most of the available whole-genome DENV sequences at GenBank have incomplete or uncorroborated 5´ and 3´ UTRs due to limitations in the conventional techniques of reverse transcription and subsequent PCR amplification, which involves the use of oligonucleotides with known consensus sequences. In addition, the lack of a poly (A) tail at the 3' UTR hinders the use of most of the commercially available kits for amplification of 3' UTR sequences based on the oligodT complementarity.

Given the critical role of the 5’ and 3’ UTRs in DENV biology, the uncertainty of these regions becomes a constraint for studies involving full-genome comparisons, identification of genetic diversity and their potential role in pathogenesis and severe disease. Here, we present a strategy for reverse transcription and PCR amplification of the complete 5' and 3' UTRs of the four DENV serotypes, its performance in positive viral isolates of dengue from passage 2 and discuss the relevance of nucleotide substitutions at the secondary structure level.

3. Objectives

3.1. General objective

To standardize and implement the characterization of the 5' and 3' UTRs of the genome of dengue virus serotypes 1, 3 and 4, as a potentially useful tool in studies of viral pathogenesis.

3.2. Specific objective

- To characterize the 5' and 3' UTRs of the genome of 15 DENV strains isolated from patients with dengue and severe dengue in Colombia, by rapid amplification of cDNA ends (RACE) and direct Sanger sequencing.
- To evaluate alterations in the secondary RNA structure of the 5' and 3' UTR of the dengue virus genome, caused by point mutations in Colombian strains.

4. Materials and methods

4.1. *Cell cultures and viral isolation*

Human sera from DENV-infected patients were diluted (1/100) in Eagle´s Minimal Essential Medium (E-MEM) supplemented with 1% HEPES, 1% $NaHCO_3$, 5% Tryptose and 2% fetal bovine serum. Subsequently, C6/36 cell monolayers were inoculated with 200 µL of each diluted serum. Viral adhesion was carried out at 28 °C in a 5% CO_2 atmosphere during 1 hour, afterwards 800 µL of supplemented E-MEM were added. The cultures were incubated at 28 ºC, 5% CO_2 atmosphere until cytopathic effect (CE) typically syncytia or cell growth arrest was observed without exceeding 14 days. A second passage for samples without showing CE was performed by transferring a 200 µL aliquot of the cell supernatant from the first passage to a fresh C6/36 monolayer. Culture supernatants were collected, cleared, aliquoted and stored at -80ºC.

4.2. *Viral RNA extraction*

The commercial QIAamp Viral RNA Mini Kit (Qiagen®, Chatsworth, CA, USA) was used for viral RNA extraction by following the manufacturer's recommendations. Briefly, a volume of 140 µl of each serum sample or cell culture supernatant (viral isolation) was mixed with 560 µl of AVL buffer containing carrier RNA. Then, 500 µl of absolute ethanol were added and the total volume transferred to a silica gel-based mini spin column and centrifuged at 8000 rpm. Subsequently, two washing steps were performed by adding 500 µl of AW1 buffer and 500 µl of AW2 to remove residual contaminants by centrifuging at 8000 and 13000 rpm, respectively. Finally, the elution was performed by the addition of 60 µL of AVE buffer to the column, incubating for 1 min at room temperature and centrifuging at 8000 rpm for 1 min. The purified RNAs were used for DENV serotyping in the virology laboratory of the National Institute of Health; using nested RT-PCR using improved oligonucleotides (Usme, Gómez, & Gallego, 2012), and stored at -70°C for subsequent cDNA synthesis.

4.3. *Primer designing for amplification of the DENV 5' and 3' untranslated regions (UTRs)*

Following the 5'/3' RACE kit 2nd generation (Roche Diagnostics GmbH, Mannheim, Germany) manufacturer´s recommendations, three specific antisense (SP1, SP2 and SP3) and two sense (SP4 and SP5) primers were designed for cDNA synthesis and PCR

amplification of the 5' and 3' UTR regions of all DENV serotypes. Primer design was carried out using the parameters described in Table 1, through the PrimerSelect module of the LaserGene® suite version 8.1 (DNASTAR Inc., Madison, WI, USA.). The genomic sequences of four DENV isolates GQ868568 (DENV-1), NC_001474 (DENV-2), GU131954 (DENV-3) and GQ868585 (DENV-4) were used as reference. Once the best primer candidates were identified, they were aligned against several DENV strains reported in South American countries (Brazil, Peru, Venezuela, Ecuador, Argentina, Chile and Paraguay), in order to assess and consider the genetic variability from this region. The primers were finally designed by following parameters such as the 3'-OH of the primer coincident with the second or first codon position (for those located in the coding region), the inclusion of degenerate sites for those nucleotide positions with representative variability of DENV strains through the South American region. Degenerate sites were introduced according to the IUPAC nucleotide ambiguity code, available at: http://www.chick.manchester.ac.uk/SiteSeer/IUPAC_codes.html.

The use of the previously designed anti-sense primers extending through the C or prM/M coding region (between positions 298 and 579, respect to each reference sequence) allowed the successful amplification of the 5' end of Colombian DENV-2 strains. Sense primers extending from the NS5 coding region and the 3'UTR (between positions 10114 and 10333, respect to each reference sequence) were successfully used for the molecular characterization of the 3' end. The PCR products were easily visualized through agarose gel electrophoresis showing a specific amplification that, allowed the direct purification and sequencing.

Table 1. Characteristics that were considered for the design of the primers for the amplification of the amplification of the 5' and 3' UTR ends.

Characteristics	Minimum	Maximum
Length primers	17 bp	24 bp
Melting	35.8ºC	62.5ºC
Stability 3' pentamer	8.5 -Kc/M	
unique sequence in the 3'	7bp	

4.4. *Reverse transcription and PCR amplification of the 5´ untranslated region*

Serotype specific anti-sense primer SP1 (12,5 pmol), 5 µL (100-200ng) of total RNA extracted from DENV infected C6/36 cell supernatants, 1 mM dNTPs, 25 U Transcriptor Reverse Transcriptase (Roche Diagnostics GmbH, Mannheim, Germany), 1X cDNA synthesis buffer and 5 U RNaseOUT (Life Technologies, Carlsbad, CA, USA) were used for the cDNA synthesis of the 5' end. The mixture was incubated for 60 min at 55°C, then, reverse transcriptase was inactivated by heating at 85°C for 5 min. The cDNA products were purified using QIAquick PCR Purification Kit (Qiagen®, Chatsworth, CA, USA) following the manufacturer´s instructions. Poly (A) tailing of the first-strand cDNA using a recombinant terminal transferase and PCR amplification of dA-tailed cDNA were performed according to the 5'/ 3' RACE kit 2nd generation manufacturer's instructions. The expected sizes of the PCR products generated with the SP2 primer were 453, 380, 448 and 477 for DENV1, 2, 3 and 4 respectively; while for the SP3 primer expected sizes were 310, 300, 298 and 321 for DENV1, 2, 3 and 4 respectively.

4.5. *Poly A tailing reverse transcription and PCR amplification of the 3´ untranslated region*

In view that RNA extracts from infected cell supernatants contain nearly exclusively positive-sense DENV genomes and the DENV genome lacks of a poly (A) tail, it is not possible to use specific forward primers (SP4 or SP5) or oligo-dT for cDNA synthesis. Therefore, the strategy consisted in the direct adenylation of positive-sense DENV RNA genomes Briefly, 15 µL of total RNA (~500ng) were poly-adenylated at the 3' end in a reaction mixture containing 0.5 units of *E. coli* poly (A) polymerase system (New England Biolabs), *E. coli* poly (A) polymerase reaction buffer (50 mM Tris-HCl, 250 mM NaCl, 10 mM MgCl2) and 1mM adenosine-5'-triphosphate (ATP) followed by incubation 37°C for 30 minutes. Afterwards, 5 µL from the previous reaction were used in cDNA synthesis reaction using high capacity cDNA reverse transcription kit (Applied Biosystems) the reaction mix included 12.5 pmol of the oligo-dT anchor primer (5'-GAC CAC GCG TAT CGA TGT CGA C-TTT TTT TTT TTT TV-3'), 1X RT-buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1mM dNTPs, 50 U MultiScribe™ reverse transcriptase and 5 units of RNaseOUT. The reaction mix was incubated at 58°C for 45 min, then 85°C for 5 min. Next, the PCR step was performed using 5 µL from the previous reaction, 37.5 pmol of oligo-dT, anchor primer 12.5

pmol of serotype-specific SP5 primer, 1.25 U *Taq* DNA polymerase (Thermo Fisher Scientific, Carlsbad, CA, USA), 1X PCR buffer, (20 mM Tris-HCl pH 8.4, 50 mM KCl), 2 mM $MgCl_2$, 1mM dNTPs and nuclease-free water for a final volume of 50 µL. The mixture was incubated at 95°C for an initial 5 min denaturation step, followed by 45 cycles (95°C for 30 sec, 50°C for 30 sec, 72°C for 90 sec), and a final extension at 72°C for 5 min. It was used in the PCR, sterile water as negative control and as a positive control it were used strains of each serotype previously typified of dengue virus. The expected sizes of the PCR products generated with the SP4 primer were 713, 563, 490 and 452, for DENV1, 2, 3 and 4, respectively, while for the SP5 primer the expected sizes were 581, 438, 389 and 389, for DENV1, 2, 3 and 4, respectively.

4.6. *DNA sequencing and sequence handling*

PCR products were purified by using the QIAquick PCR purification kit (Qiagen®, Chatsworth, CA, USA) and the expected sizes confirmed by agarose gel electrophoresis. Around 40 ng of each purified amplicon were added to a sequencing reaction mixture containing 3 µL of BigDye® terminator cycle sequencing v3.1 (Applied Biosystems, Carlsbad, CA, USA), 3.2 pmol of the serotype-specific oligonucleotide, and 2 µL of 5X sequencing buffer (400 mM Tris-HCl, 10mM $MgCI_2$), in a final volume of 10 µL. The thermal profile consisted of an initial denaturation step at 96°C for 1 min, followed by 25 cycles (96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min); The DNA fragments were purified through the BigDye® Xterminator purification kit (Applied Biosystems, Carlsbad, CA, USA), and subsequently processed by the ABI 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The electropherograms were visualized, edited and assembled through the SeqMan module of LaserGene® v8.1 (DNASTAR Inc., Madison, WI, USA.).

4.7. *Sequence alignment and evolutionarily-conserved RNA structure prediction*

Homologous sequences were aligned through the ClustalW algorithm implemented in MEGA version 7 (Kumar, Stecher, & Tamura, 2016). For comparison, some available complete genomes of the different serotypes of DENV circulating in South America were included in the analysis. The Basic Local Alignment Search Tool (BLAST) was used to verify the similarity of the obtained sequences in the present study. The Colombian sequences corresponding to the 5′ and 3′ UTR of each DENV serotype were used for prediction of secondary structures and the identification of evolutionarily-conserved RNA elements, through the use of RNAstructure and RNAz web servers, available at http://rna.urmc.rochester.edu/RNAstructureWeb/ and http://rna.tbi.univie.ac.at/cgi-

bin/RNAz/RNAz.cgi, respectively; on the other hand, the RNAfold software was used as a tool to have a general overview of the pertaining secondary structures at the DENV UTR, available at http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi.

5. Results

5.1. *Amplification of the 5' UTR of all DENV serotypes*

Following the strategy described in Materials and Methods, serotype-specific primers (SP1, SP2 and SP3) were designed for amplification of the 5' end of the DENV genome (Table 2). The 5' UTR of Colombian strains corresponding to the four DENV serotypes was successfully amplified by using the standard RACE approach that involved the polyadenylation of the cDNA generated with the primer SP1 (Figure 1a), However, in the first round of PCR amplification (with serotype-specific primer SP2), bands of the expected size were only observed for isolates of DENV-2 and -3. While in the second PCR round (with serotype-specific primer SP3), bands of the expected size were observed for all DENV serotypes. (Figure 2). The cycling conditions consisted of an initial denaturation at 95°C for 2 min, 40 cycles of denaturation (95°C for 20 sec), annealing (30 sec at 58°C for DENV-1 and DENV-3) or 50°C for DENV-4), and extension (72°C for 30 sec), with a final extension at 72°C for 7 min.

Table 2. Serotype-specific primers used for amplification and sequencing of the 5' and 3' ends of the DENV genome.

Serotype	Primer name	Orientation	Region	Genomic position [a]	Sequence (5´- 3´)[b]	TM
DENV-1	DENV1_SP1	Antisense	5´	560-579	CCGGGGGCATTTGTAGGTCA	59.1ºC
	DENV1_SP2	Antisense	5´	431-453	TATCATGTGTGGCTCTCCYCCTC	54.7ºC
	DENV1_SP3	Antisense	5´	287-310	CTTTGATYGCTCCATTCTTCTTGA	56.5ºC
	DENV1_SP4	sense	3´	9949 – 9970	CACCAATGGATGACAACAGAAG	51.9ºC
	DENV1_SP5	sense	3´	10119 - 10140	CACCTGGGCCACCAACATACAA	59.1ºC
DENV-2[c]	DENV2_SP4	Sense	3´	10114-10137	AGAACATCCAAACAGCAATAAATC	59.0ºC
	DENV2_SP5	Sense	3´	10238-10262	AAGGGAAGAGGAAGAGGCAGGTGT	61.8ºC
DENV-3	DENV1_SP1	Antisense	5´	560-579	GGTRATRTGGGGGCATTTGTAAG	53.6ºC
	DENV1_SP2	Antisense	5´	429-448	CGGCTCTCCATCTCGTGAAG	54.7ºC
	DENV1_SP3	Antisense	5´	276-298	CGACTTCTTGAAGGTTCCCCATC	57.7ºC
	DENV1_SP4	sense	3´	10210-10233	GAAGGAGGAGGARTCGGAGGG	55.7ºC
	DENV1_SP5	sense	3´	10311-10333	GCCTGTGAGCCCCGTCTAAG	56.1ºC
DENV-4	DENV1_SP1	Antisense	5´	496-517	TTGTTGATCCCCTCTGTTGTYT	52.6ºC
	DENV1_SP2	Antisense	5´	454-477	CCCTTTCATGTTTTGCCACTATCA	56.9ºC
	DENV1_SP3	Antisense	5´	298-321	GTGGGATGGAAAGRACTCGCA	58.5ºC
	DENV1_SP4	sense	3´	10302-10320	GCAAACCGTGCTGCCTGTA	54.5°C
	DENV1_SP5	sense	3´	10098-10120	GGACTTTCTTCYAGAGCCACCTG	53.2ºC

[a] Genomic positions were estimated for every DENV serotype according to the sequences available in GenBank with accession numbers: GQ868568 (DENV-1), NC_001474 (DENV-2) GU131954 (DENV-3) and GQ868585 (DENV-4). [b] Degenerate sites were included by following the IUPAC nucleotide ambiguity code. [c] DENV-2 primers were designed in a previous study by Laiton-Donato 2016.

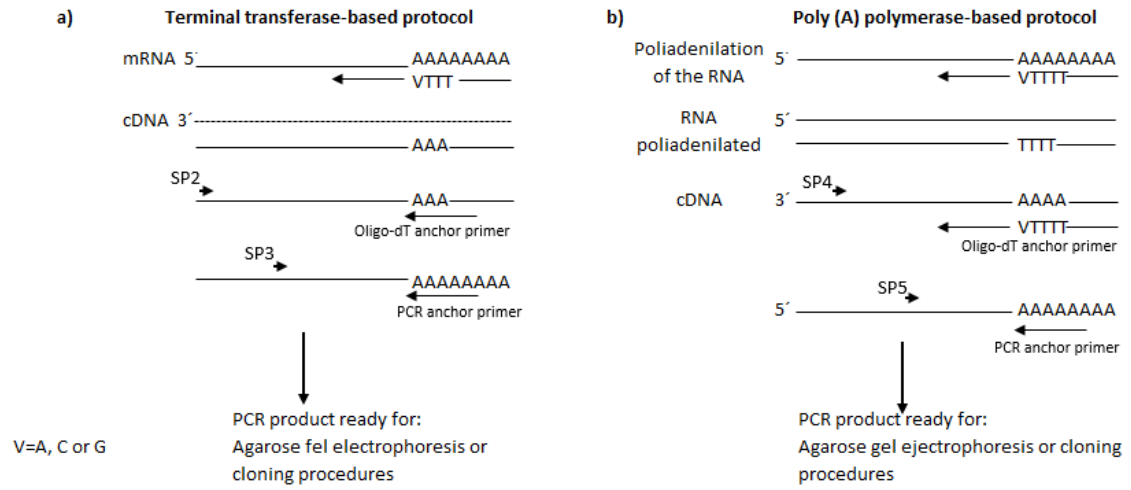

Fig. 1. Strategy for polyadenylation, cDNA synthesis and PCR amplification of the 5' and 3' ends of the DENV genome. a). Terminal transferase-based protocol for amplification of the 5´ end b). Poly (A) polymerase-based protocol for amplification of the 3´ end. V: A, C or G. adapted from the 5'/3' RACE kit 2nd generation (Roche Diagnostics GmbH, Mannheim, Germany).

5.2. *Amplification of the 3´ UTR of all DENV serotypes*

For cell-free supernatants of infected C6/36 cells, the standard RACE approach was not expected to be an efficient method for the characterization of the DENV 3' UTR, because of the exclusive presence of positive-sense RNA genomes in the secreted viral particles. A first approach to overcome this limitation, consisted in applying the RACE strategy to total RNA extracts obtained from cell lysates, in which the presence of negative-sense viral RNA within dsRNA and replicative intermediates is expected (Chu & Westaway, 1985). This strategy was not successful (data not shown), possibly due to the asymmetric replication of the DENV genome, and therefore the low abundance of negative-sense viral RNA strands (Diamond, Edgil, Roberts, Lu, & Harris, 2000). A second and novel approach consisted in the poly-adenylation of the positive-sense viral RNA by using the *E. coli* poly (A) polymerase, and subsequent oligo-dT hybridization on the artificially generated poly (A) tail (Figure 1b), which demonstrated to be successful for specific amplification of the 3' UTR of DENV -2, -3 and -4. (Figure 3). The cycling conditions that were standardized for each serotype consisted of an initial denaturation of 95°C for 2 min, 30 cycles of denaturation at 95°C for 20 sec, annealing for 30 sec at 52°C for DENV-2, 61°C for DENV-3 and 52°C for DENV-4, and extension at 72°C for 30 sec), with a final extension at 72°C for 7 min, in case of DENV -3 and -4 were 40 cycles. DENV-1 strains were not.

At the best of our knowledge, this novel poly (A) polymerase-based approach has not been previously used for molecular characterization of the 3' UTR of positive-sense viral RNA genomes.

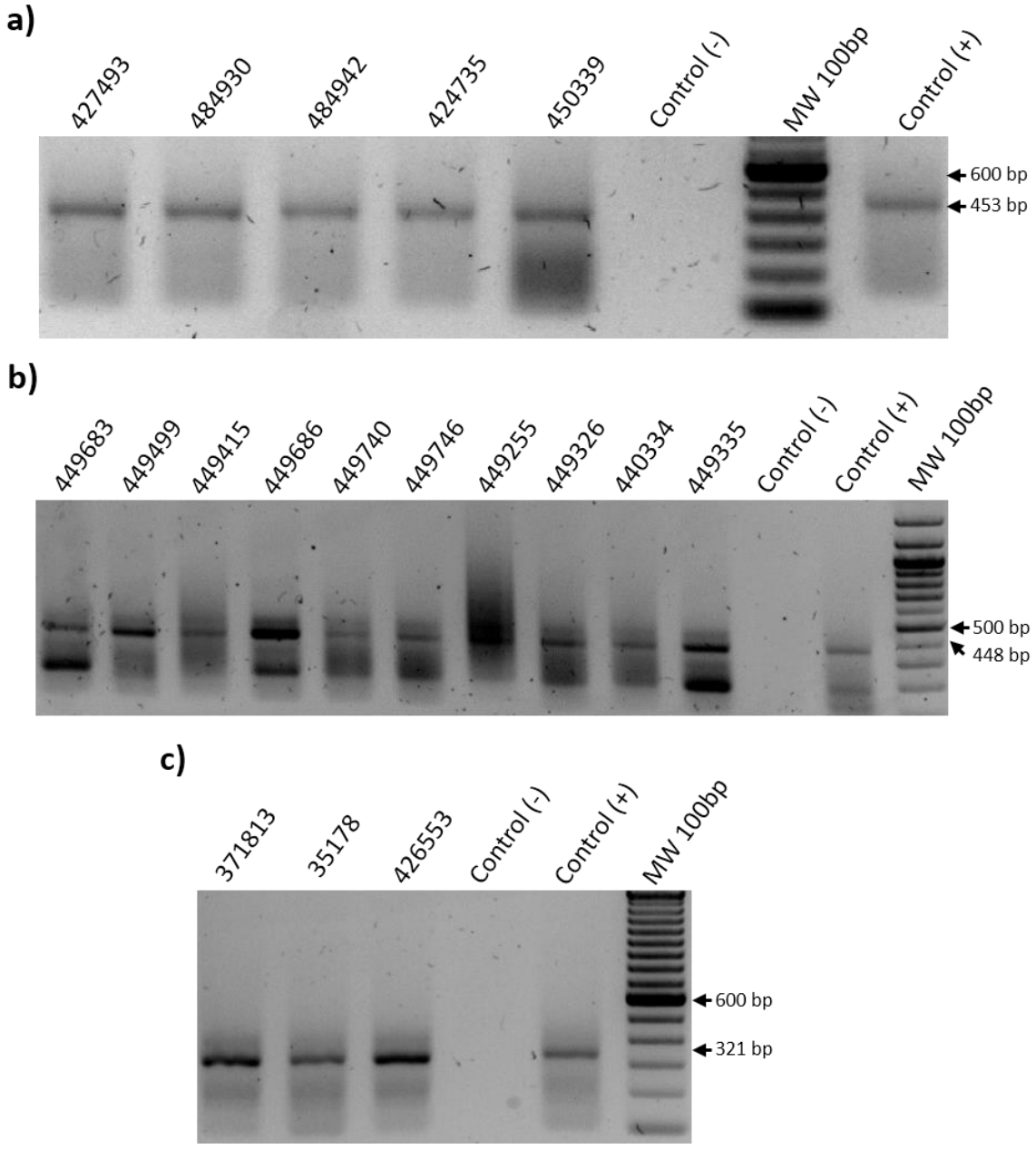

Fig. 2. PCR amplification of the 5´end of dengue virus serotypes 1, 3 and 4. **a)**, **b)** and **c)** Nested PCR using SP2 or SP3 and oligodT anchor primers for successful amplification of DENV-1, -3 and -4 strains, respectively. Previously characterized DENV strains were used as positive controls. The PCR product was 453, 448 and 321 bp in length for DENV-1, -3 and -4, respectively. MW: 100 bp DNA ladder (Invitrogen) were used in **a)** and **b)** with 600 bp and the PCR product of each serotype marked; on the other hand, **c)** 100 bp DNA ladder (Thermo Fisher) was used with 500 bp and the PCR product marked.

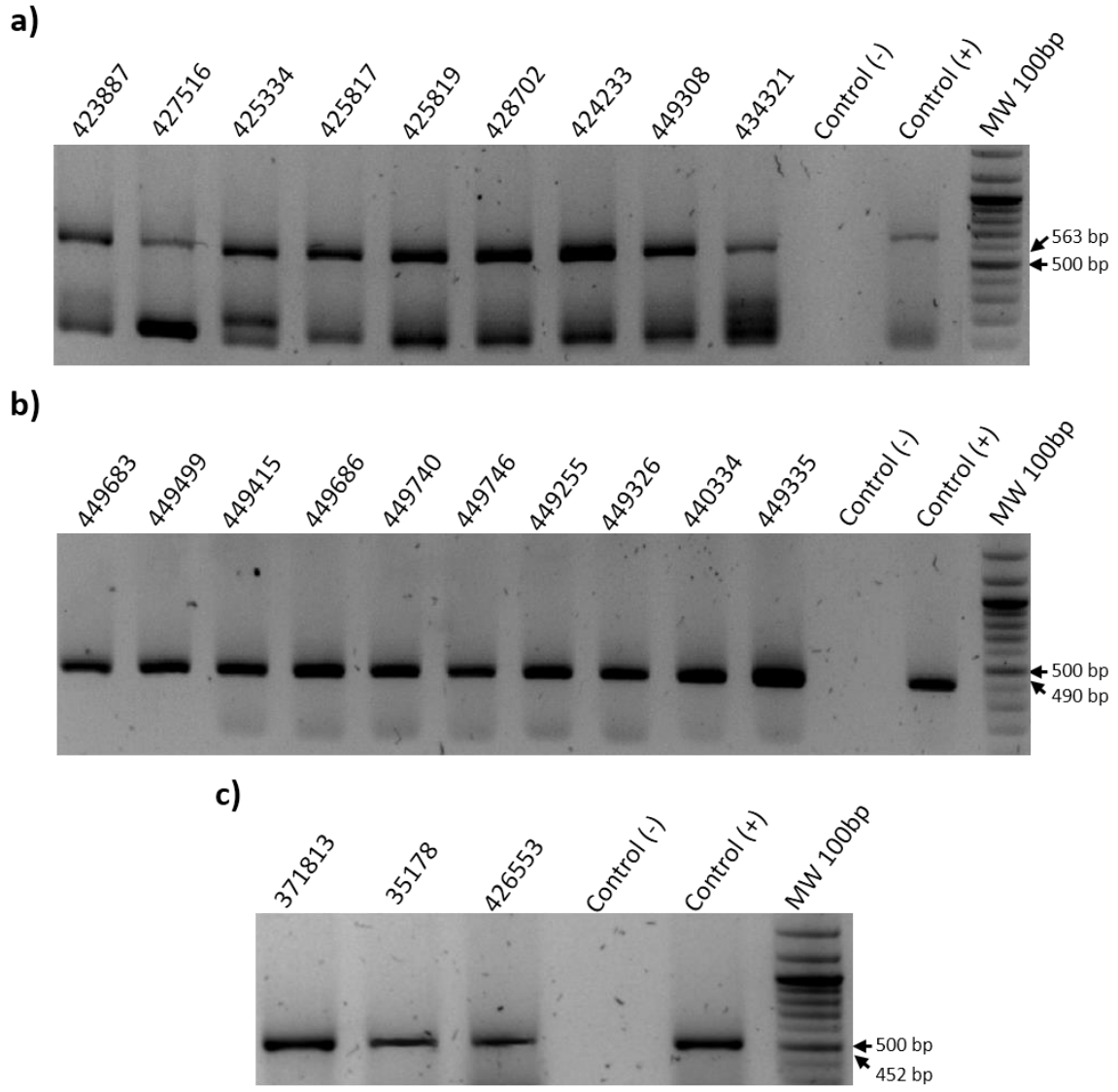

Fig. 3. PCR amplification of the 3´end of dengue virus serotypes 2, 3 and 4. **a)**, **b)** and **c)**. Nested PCR using SP4 and oligodT anchor primers for successful amplification of DENV-2, -3 and -4 strains, respectively. Previously characterized DENV strains were used as positive controls. The PCR product was 563, 490 and 452 bp in length for DENV-2, -3 and -4, respectively. MW: GeneRuler 100 bp DNA ladder (Thermo Fisher) with 500 bp and the PCR product of each serotype marked.

5.3. *Molecular characterization of the 5' and 3' ends of the dengue virus genome*

The DENV 5´ and 3´ end sequences were obtained by Sanger sequencing with specific primers (SP2, SP3, SP4 or SP5), as no good quality sequences were obtained with oligo-dT primer as expected from the variable size of the poly (A) tract among DNA molecules. A total of 31 isolates from Colombian clinical samples were successfully sequenced, 26 isolates belonged to patients with dengue without warning signs (3 from DENV-1, 10 from

DENV-2, 10 from DENV-3 and 3 from DENV-4) and 5 from severe dengue patients (all from DENV-2) (Table 3). The electropherograms allowed to confirm the presence of the artificially generated poly (A) tail (Figure 4). Currently available full-length genomes of Colombian DENV isolates available at GenBank have incomplete or uncorroborated 5' and 3´ ends, typically lacking the first or last ~20 nt at the 5´ and 3´ ends, respectively. Using the BLAST alignment tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) the sequences obtained in the present study matched with other DENV strains reported in American countries (Cuba, Haiti, Brazil, Peru, Venezuela, Ecuador, French Guiana, Argentina, Chile and Paraguay). Even though our approach yielded sequences of the 5' and 3' ends for most of the tested isolates, four isolates showed a shorter amplicon size at the 5´end characterized by the presence of the poly (A) tract at the position 134 (relative to the reference sequence NC_001474). Interestingly the isolates that showed this behavior also, showed a noticeable drop in the fluorescence signal in the electropherograms, from that position to the beginning (position 1 of the viral genome).

Table 3. List of DENV-1, -2, -3 and -4 strains included in the study and associated clinical characteristics.

Serotype	Code	Strains	Year	Department	UTR sequenced	Clinical classification	Access number
	424735	58	2013	META	5´	DF	
DENV 1	427493	61	2013	TOLIMA	5´	DF	
	450339	69	2015	CUNDINAMARCA	5´	DF	
	423887	12	2013	PUTUMAYO	3´	DF	
	427516	28	2013	CALDAS	3´	DF	
	425334	41	2013	PUTUMAYO	3´	DF	
	425817	44	2013	TOLIMA	3´	DF	
DENV 2	425819	46	3013	CUNDINAMARCA	3´	DF	
	428702	56	2014	TOLIMA	3´	DF	
	424233	59	2013	BOYACÁ	5´ / 3´	DF	
	449418	62	2015	TOLIMA	5´	DF	
	449308	66	2015	HUILA	5´ / 3´	DHF/mortal	
	449618	67	2015	HUILA	5´	DF	
	449510	68	2015	PUTUMAYO	5´	DF	
	434321	156	2014	META	3´	DF	
	449683	57	2015	RISARALDA	5´ / 3´	DF	
	449499	60	2015	RISARALDA	5´ / 3´	DF	
	449415	63	2015	PUTUMAYO	5´ / 3´	DF	
	449740	81	2015	SUCRE	5´ / 3´	DF	
DENV 3	449746	82	2015	RISARALDA	5´ / 3´	DF	
	449255	84	2015	QUINDÍO	5´ / 3´	DF	
	449326	85	2015	RISARALDA	5´ / 3´	DF	
	449334	86	2015	BOYACÁ	5´ / 3´	DF	
	449335	87	2015	BOYACÁ	5´ / 3´	DF	

	371813	88		5′ / 3′
DENV 4	37178	89		5′ / 3′
	426553	90		5′ / 3′

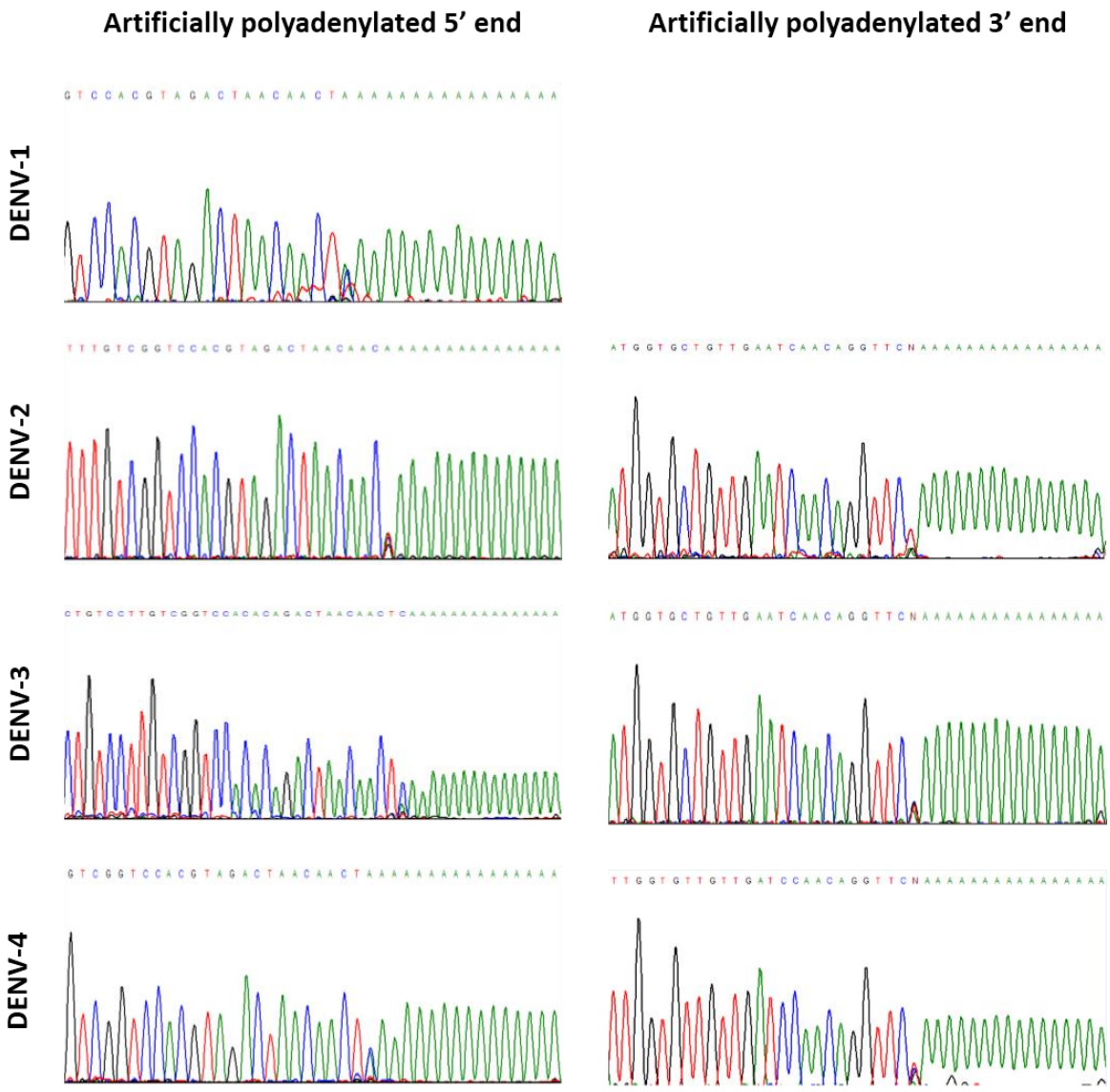

Fig.4. Artificial polyadenylation at the 5' and 3' ends of the DENV genome. Electropherograms of the 5′ and 3′ends of representative strains of DENV-1 to DENV-4. The 5’ and 3’ ends were sequenced by using specific reverse and forward primer, respectively.

5.4. *Evolutionarily conserved structural elements at the 5´ and 3´ untranslated regions of dengue virus*

The two highly conserved secondary structures corresponding to the stem-loop A (SLA) and SLB were identified at the 5´ UTR, as well as the conserved RNA elements 5' UAR and 5' CS. In addition, it was possible to identify and the cHP element extending to the capsid-coding region (Figure 5A); On the other hand, at the 3' UTR the 3´ SL, DB1 and DB2 secondary structures were found with their respective conserved sequences RCS2 and CS2; in this region we also found the variable region and the conserved 3' UAR and 3' CS (Figure 5B).

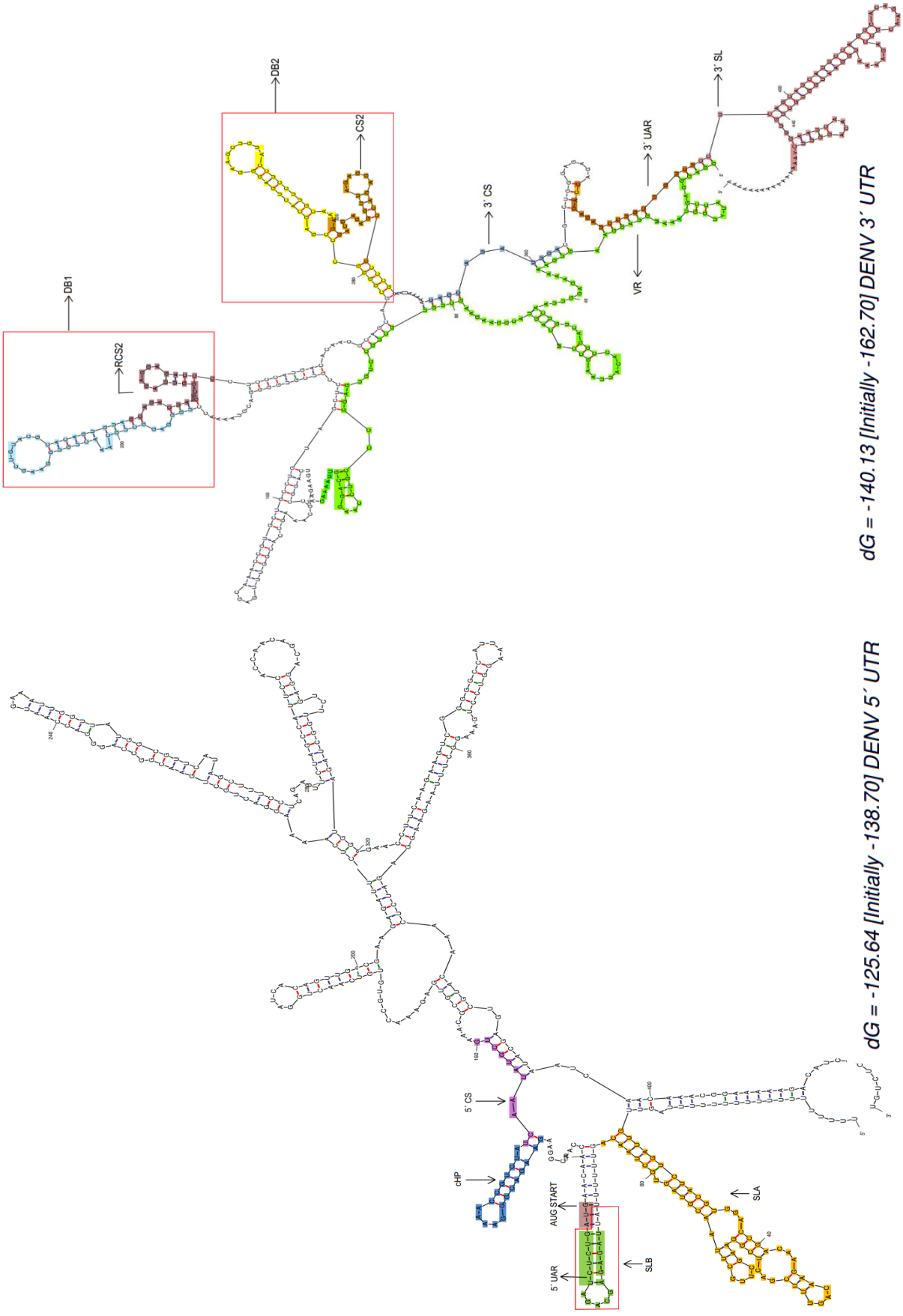

FIG.5. RNA structure elements at the DENV 5 ' and 3' UTR. A) Elements present at the 3' UTR. B) Elements at the 5´ UTR.

At the 5' UTR of DENV, the structure analyzes of the DENV-2 5´end of the strains that showed the late poly-A tail and which generated shorter sequences, were not able to obtain the secondary structures present in the 5´ end, since, when analyzing the late poly (A) tail was found in the position 134 which is after SLA and SLB structures, curiously, the position of the late poly (A) was the same in all four strains, although, , when the amplification test was repeated with a high fidelity *Taq* polymerase, bands with a smaller size than expected were obtained. On the other hand, two thermodynamically stable secondary structures were identified in serotypes 1, 3 and 4.The first structure corresponds to the SLA element and the second structure corresponds to the SLB element (Figure 6).

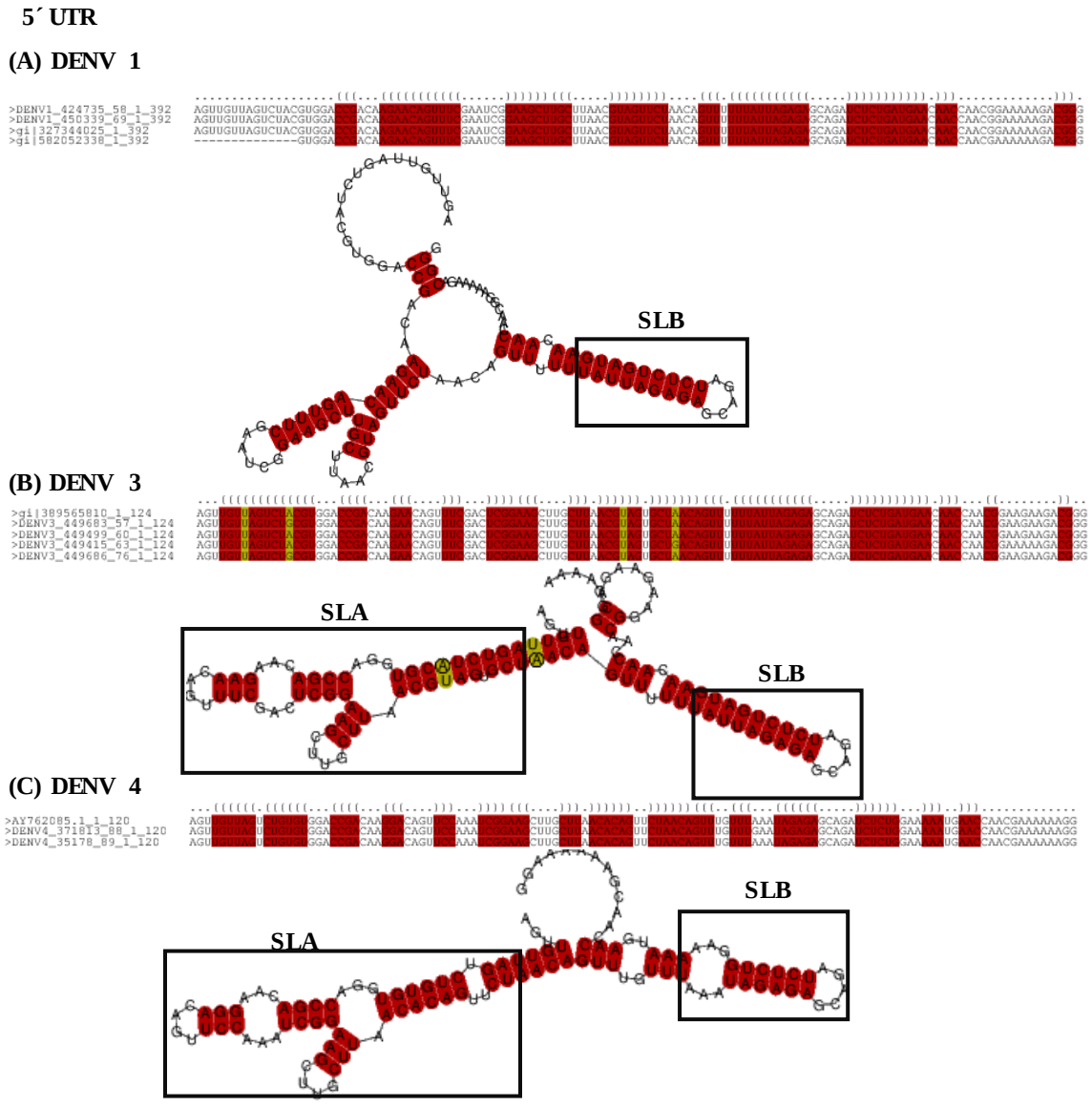

Fig 6. Alignment and secondary structures present in the 5'utr region of DENV. A) alignment and structure of the SLA and SLB elements of DENV 1, B) alignment and structure of SLA and SLB elements of DENV 3 and C) alignment and structure of SLA and SLB elements of DENV 4.

On the other hand, in the 3'UTR region, the DB2 and 3'SL elements were identified for serotypes 2, 3 and 4 (Figure 7). For all elements analyzed in both the 5'UTR region and the 3'UTR, different parameters were determined that are related to their thermodynamic stability Table 4.

3´ UTR

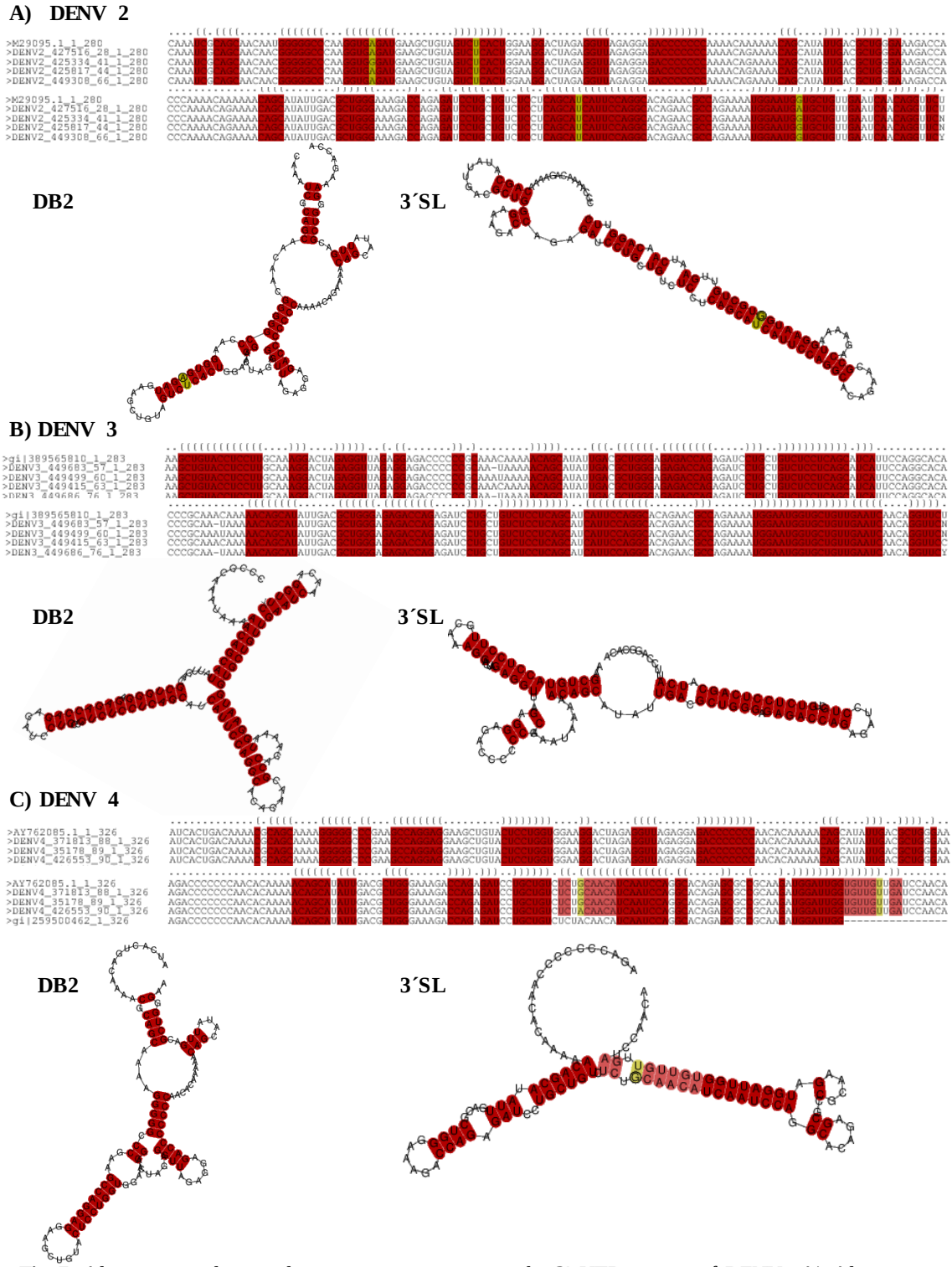

Fig 7. Alignment and secondary structures present in the 3' UTR region of DENV. A) Alignment and structure of the DB2 and 3´SL elements of DENV 2, B) Alignment and structure of DB2 and 3´SL elements of DENV 3 and C) Alignment and structure of DB2 and 3´SL elements of DENV 4.

Table 4.

Thermodynamic stability of the secondary structures present in the 5' and 3' UTR of DENV -1, -2, -3 and -4.

Parameters	DENV 1		DENV 2		DENV 3				DENV 4			
	SLA	SLB	DB2	3´SL	SLA	SLB	DB2	3´SL	SLA	SLB	DB2	3´SL
Location	0-120	0-120	80-200	160-280	0-124	0-124	120-240	163-283	0-120	0-120	120-240	200-320
Length	120	120	120	120	120	120	120	120	120	120	120	120
Mean pairwise identity	93.75	93.75	99.00	98.67	98.87	98.87	99.0	98.25	99.44	99.44	100.00	94.17
Covariance contribution	0.00	0.00	-0.16	-0.16	-0.40	-0.40	0.00	0.00	0.00	0.00	0.00	1.48
Mean z-score	-2.27	-2.27	-1.64	-3.89	-2.31	-2.31	-1.27	-3.99	-2.90	-2.90	-2.38	-2.28
Structure conservation index	0.84	0.84	0.99	0.99	1.01	1.01	1.00	1.00	1.00	1.00	1.00	0.94

5.5. *Coverage at the 5' and 3' ends of the DENV genome in previously reported sequences*

In order to assess the coverage at the 5’ and 3’ ends, previously reported sequences of DENV strains circulating in neighboring countries (Brazil, Peru, Venezuela, Ecuador, Argentina, Chile and Paraguay) were aligned with those of the present study. At least 20 nucleotides are missing in the previously published sequences and were successfully sequenced through the methods described here (Figure 8). At the 3’ end, the last nucleotide is called “N” to designate a variable position, this is a consequence of using a degenerate oligo-dT anchor primer containing A, C or G at the 3’ end (Figure 8B). At the 5’ end, the “N” is also present at the -1 position, therefore, it does not affect the sequence of interest (Figure 8A).

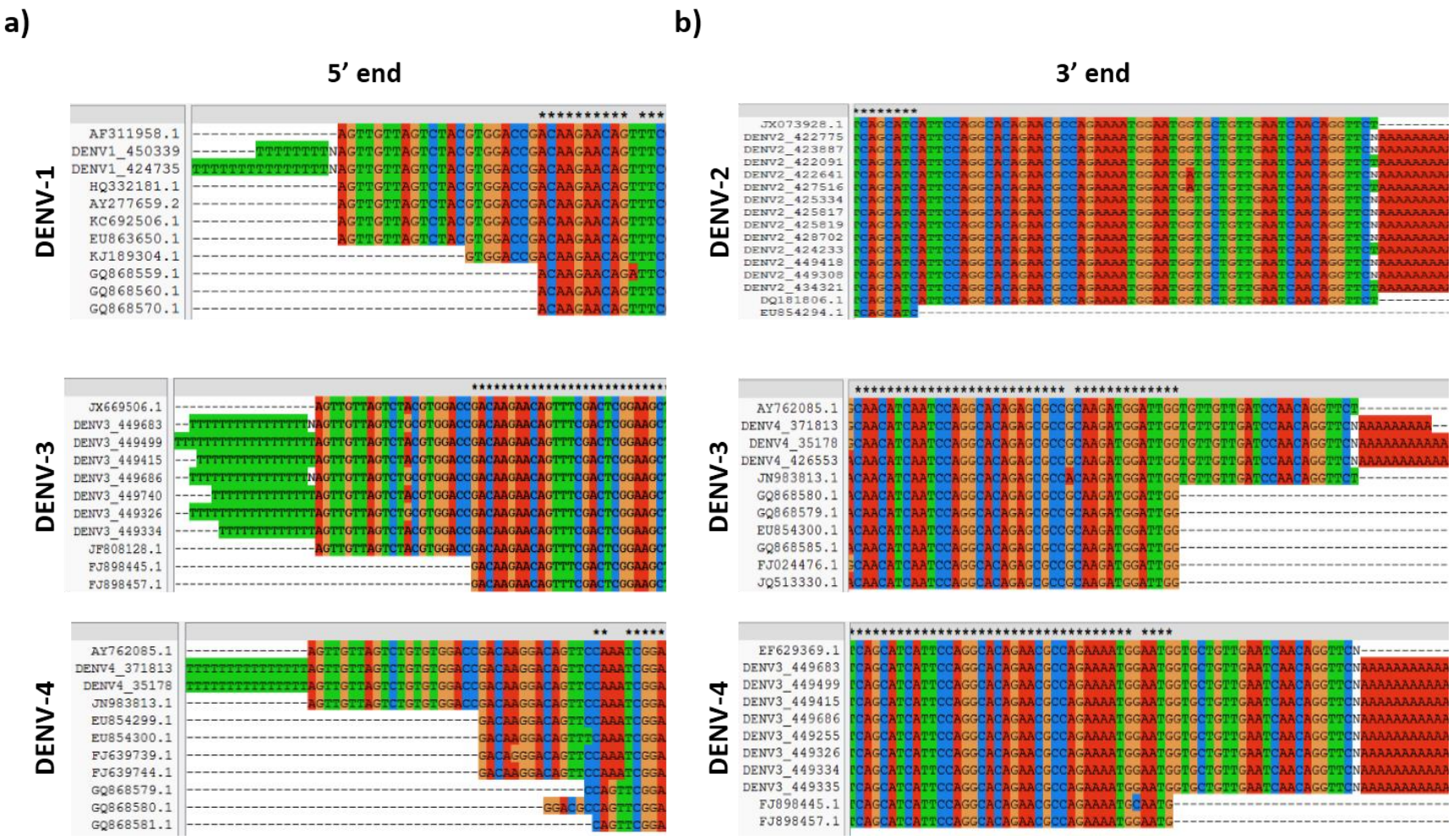

Fig.8. Alignment of sequences obtained with reported in GenBank database. a) Sequences belonging to the end amplification of the 5' UTR region. b) Sequences belonging to the amplification of the 3' UTR region end.

6. Discussion

During the last two decades, the intense research in fundamental biology of DENV and other closely-related flaviviruses has allowed deciphering the role or function of almost all viral proteins during the virus life cycle. These studies have also allowed the identification of several genome features that explain some of the phenotypic differences at the virus replication or virus-host interaction and pathogenesis levels. Genome differences include the presence/absence of nucleotide substitutions and insertion/deletions in the coding and the untranslated regions. It has been shown that the replication capacity of a virus is indispensable at the time of deciphering key elements in the pathogenesis and virulence. The 5´and 3´ UTR could harbor some important determinants of virulence (Leitmeyer *et al.*, 1999), for this reason, analyzing these regions through complete sequencing is mandatory.

The DENV RNA-dependent RNA polymerase endows the DENV genome with a high mutation rate ranging from $4.5x\ 10^{-4}$ substitutions per site every year (s/s/y) for DENV-1 to $9x10^{-4}$ s/s/y for DENV-3, with some differences according to the serotype (Falcón-Lezama *et al.*, 2009). The unavoidable accumulation of genetic variation in the whole DENV genome suppose a challenge for sequence-based molecular methods and therefore justify the rational design of degenerated oligonucleotides that theoretically cover the existent genetic variability and perform well for a longer period of mutation accumulation. Here, we used the PrimerSelect module of the LaserGene suite, which integrates the Nearest Neighbor algorithm for ΔG and melting temperature (Tm, the temperature at which half of the oligonucleotides have hybridized with the sequence) calculation. Using this algorithm, it was possible to design primers that worked well at the time of amplifying and sequencing the 5' and 3' ends of the DENV genome. All oligonucleotide candidates were analyzed through sequence alignment to cover the existing variability of DENV strains across the region and to avoid the use of critical primer positions according to the evolutionary behavior (e.g. codon position); the criteria that were used in this design of primers have already been validated previously in the study carried out by (Usme *et al.*, 2012)

In this work we demonstrated the successful cDNA synthesis and PCR amplification of several strains of the different DENV serotypes through the use of specific and sometimes degenerated oligonucleotides. The strategy consisted in following the standard terminal transferase-based RACE protocol for the 5’ end characterization.

The original RACE protocol for the 3' end was intended to work directly with polyadenylated RNA (e.g. messenger RNA), so in the case of DENV it was necessary to attach a poly (A) tail at this end. Therefore, a novel approach for the 3' end characterization was implemented using the *E. coli* poly (A) polymerase, which was based on the direct polyadenylation of the positive-sense viral RNA genomes previously extracted from supernatants of infected cells. The artificially generated poly (A) tail provided a target for the oligo-dT hybridization during the cDNA synthesis and subsequent PCR amplification following the standard RACE protocol. High quality electropherograms evidenced the successful incorporation of a poly (A) tract to the viral genome and the complete resolution of the 3' end sequence of the analyzes DENV strains of serotypes 2, 3 and 4. There were technical problems for PCR amplification of the 3' end of DENV-1, however, it was not possible to attribute the cause to the primer design, because several oligonucleotide sets were unsuccessfully used.

The technical approach presented here could be routinely used as a complement to full-genome sequencing through different methods, by increasing the coverage at the genome ends and allowing more accurate association studies to phenotypic characteristics.

The vast majority of Colombian DENV full-length genomes available at GenBank lack the 5' and 3' ends of the UTR, due to the limitations imposed by current RNA genome amplification techniques by reverse transcription and PCR, which involves the use of oligonucleotides with previously known sequences during cDNA synthesis or PCR amplification; On the other hand, it has not been possible to establish an optimal method for its amplification without the risk of losing the end (Christenbury *et al.*, 2010). Next generation sequencing (NGS) technologies such as reversible terminator sequencing-by-synthesis by Illumina have shown to work well for the characterization of full-length RNA genomes including the UTRs with a level of detail for analyzing viral quasispecies and the presence of multiple genotype variants in a single sample, however, the computational methods for assembling Illumina short reads and solve homopolymeric regions at the DENV UTRs are still challenging (Caraballo Cortes *et al.*, 2016). Hence, the methods described here offers a simple, straightforward and cost-effective way to characterize the most representative UTR sequences of any DENV serotype.

It has been shown that there are factors associated with the virus that contribute in a differential way to the development of the disease, known as determinants of virulence.

These factors have been associated with the serotype and genotype of the virus and identified as with point mutations that directly affect the viral fitness and therefore the levels of viremia in the human host (Vaughn *et al.*, 2000); Other studies suggest that virulence determinants are present randomly throughout the DENV genome (Daira Camacho *et al.*, 2009; Daría Camacho, Ferrer, Tenorio, Franco, & Comach, 2012), associated with the envelope gene or located at the 5' and 3' UTR (Leitmeyer *et al.*, 1999) where they are involved in the initiation of translation (You & Padmanabhan, 1999), and in the regulation of viral replication; (Alvarez, De Lella Ezcurra, Fucito, & Gamarnik, 2005; Daira Camacho *et al.*, 2009; Daría Camacho *et al.*, 2012; Rico-Hesse *et al.*, 1997). It has been considered that the more virulent strains are those with greater replicative capacity (Vaughn *et al.*, 2000), therefore, the *in vitro* characterization becomes an indirect way of evaluating its capacity to cause disease.

In this work we modified the RACE protocol since, as already mentioned, this protocol works directly with messenger RNA so in the case of DENV it was necessary to attach a poly A tail at the 3' UTR end, with the enzyme *E. coli* Poly A polymerase was able to provide a site for the synthesis of the cDNA using an Oligo-dT and in this way when binding by base pairing with the Poly A tail it was possible to obtain the end of the non-coding 3' UTR region. Due to the successful incorporation of the adenine tail, it was possible to implement the RACE protocol and in this way to amplify and sequence the complete ends of the DENV.

A deletion of 11 nucleotides in the variable region (VR) was observed in 3 strains of DENV-3; when comparing these sequences from Brazil, Nicaragua and Singapore, but not in Colombian strains; Previous studies have demonstrated deletions and nucleotide variations in the (VR) within the same serotype (Aquino *et al.*, 2006; Roche, Cassar, Laille, & Murgue, 2007); In spite of these observations, a high conservation was also found in the 5'UAR, 5'CS elements present in the 5' UTR region and in the case of the 3' UTR two variations were observed in the elements DB1 and DB2; On the other hand, the elements 3'SL, 3'UAR, 3'CS were the most conserved, as expected, since these elements belong to domain III, which has been considered the most conserved region at the 3´UTR. (Iglesias & Gamarnik, 2011).

When comparing the sequences of the elements present in the UTRs that were obtained in this work with those found in the work of (Paranjape & Harris, 2010), it was found that both the SLB and 3'SL elements as well as the sequences 5'UAR, 5'CS, 3'CS and 3'UAR remained preserved; While in the elements SLA, cHP, and DB1 variations were found, just as in the CS2 sequence present in the DB2 element; on the other hand, with the RNAz software it was

found that the structures analyzed for the 5' UTR region have an identity of ~ 97.18% at the nucleotide level, in addition, two secondary structures denominated SLA and SLB were shown *in silico* (Gebhard *et al.*, 2011); In the 3' UTR region an identity of 98.18% was found, in this region two structures denominated DB2 and 3'SL were evidenced (Gebhard *et al.*, 2011). Any mutation in regions that are highly structured such as UTRs could lead to alterations in the structure and that previous studies are known to be very important at functional level; this would explain why, no conformational changes were found in the secondary structures encountered, or other additional structures.

At the time of analyzing the sequences of the 5' UTR ends of the four serotypes we found a double peak in the sequences and a premature adenine tail; (Añez, Heisey, Volkova, & Rios, 2016) it was thought to be the result of the existence of a heterogeneous viral population that contains two master sequences whit almost the same frequency and different length due to insertion/deletion mutations.

The study presents a novel technical approach to the DENV genome ends characterization, which could contribute to the extension of the frontier of knowledge through the identification of genetic determinants of pathogenicity that lead to severe manifestations of dengue.

7. Conclusions

The methodology was successful for the amplification of the 5' and 3' UTR ends of the strains to be analyzed, a total of 31 Colombian DENV isolates were identified, 26 from dengue patients with no warning signs (3 from DENV-1, 10 from DENV-2, 10 from DENV-3 and 3 from DENV-4) and 5 from severe dengue DENV-2); of which, it was possible to solve the complete ends of the DENV genome and to observe that although variations were found in some sequential structures, they did not generate any change in the structure.

This study will provide new knowledge and an important tool for obtaining information from the ends of the viral genome for the evaluation of the effect of mutations on the secondary structure of RNA and its potential impact on viral replication and pathogenesis.

8. Funding

This work was supported by COLCIENCIAS (grant 210465740977 to J.A. Usme-Ciro) and the Colombian National Institute of Health.

9. Acknowledgments

The authors thank the National Laboratory Network for routine virological surveillance of dengue in Colombia; the Sequencing and Genomics Unit team; and Angélica Rico and Lissethe Pardo for providing samples with confirmed diagnosis and serotyping.

10. References

Alvarez, D. E., De Lella Ezcurra, A. L., Fucito, S., & Gamarnik, A. V. (2005). Role of RNA structures present at the 3'UTR of dengue virus on translation, RNA synthesis, and viral replication. *Virology, 339*(2), 200-212.

Aquino, V. H., Anatriello, E., Goncalves, P. F., EV, D. A. S., Vasconcelos, P. F., Vieira, D. S., Figueiredo, L. T. (2006). Molecular epidemiology of dengue type 3 virus in Brazil and Paraguay, 2002-2004. *Am J Trop Med Hyg, 75*(4), 710-715.

Añez, G., Heisey, D. A., Volkova, E., & Rios, M. (2016). Complete Genome Sequences of Dengue Virus Type 1 to 4 Strains Used for the Development of CBER/FDA RNA Reference Reagents and WHO International Standard Candidates for Nucleic Acid Testing. *Genome Announc, 4*(1).

Brathwaite Dick, O., San Martín, J. L., Montoya, R. H., del Diego, J., Zambrano, B., & Dayan, G. H. (2012). The History of Dengue Outbreaks in the Americas. *Am J Trop Med Hyg, 87*(4), 584-593.

Bäck, A. T., & Lundkvist, Å. (2013). Dengue viruses – an overview. In *Infect Ecol Epidemiol* (Vol. 3).

Camacho, D., Bracho, M., Rodríguez, F., Díaz, L., Guzmán, M., & Comach, G. (2009). Propiedades biológicas de cepas venezolanas de DENV-2 aisladas de pacientes con fiebre de dengue y fiebre hemorrágica del dengue. *Revista Cubana de Medicina Tropical, 61*(3), 259-268.

Camacho, D., Ferrer, E., Tenorio, A., Franco, L., & Comach, G. (2012). Epidemiología molecular de los virus Dengue. *Boletín de Malariología y Salud Ambiental, 52*(1), 1-13.

Caraballo Cortes, K., Bukowska-Osko, I., Pawelczyk, A., Perlejewski, K., Ploski, R., Lechowicz, U., Radkowski, M. (2016). Next-Generation Sequencing of 5' Untranslated Region of Hepatitis C Virus in Search of Minor Viral Variant in a Patient Who Revealed New Genotype While on Antiviral Treatment. *Adv Exp Med Biol, 885*, 11-23.

Chiu, W.-W., Kinney, R. M., & Dreher, T. W. (2005). Control of Translation by the 5′- and 3′-Terminal Regions of the Dengue Virus Genome.

Christenbury, J. G., Aw, P. P., Ong, S. H., Schreiber, M. J., Chow, A., Gubler, D. J., Hibberd, M. L. (2010). A method for full genome sequencing of all four serotypes of the dengue virus. *J Virol Methods, 169*(1), 202-206.

Chu, P. W., & Westaway, E. G. (1985). Replication strategy of Kunjin virus: evidence for recycling role of replicative form RNA as template in semiconservative and asymmetric replication. *Virology, 140*(1), 68-79.

Diamond, M. S., Edgil, D., Roberts, T. G., Lu, B., & Harris, E. (2000). Infection of Human Cells by Dengue Virus Is Modulated by Different Cell Types and Viral Strains.

Falcón-Lezama, J., Servicios de Salud del Estado de Morelos, C., México, Sánchez-Burgos, G. G., CMN Ignacio García Téllez, M., México, Ramos-Castañeda, J., & INSP, C., México. (2009). Population genetics of dengue virus and transmission of dengue fever. *Salud pública Méx, 51*.

Filomatori, C. V., Iglesias, N. G., Villordo, S. M., Alvarez, D. E., & Gamarnik, A. V. (2011). RNA Sequences and Structures Required for the Recruitment and Activity of the Dengue Virus Polymerase*. In *J Biol Chem* (Vol. 286, pp. 6929-6939).

Friebe, P., & Harris, E. (2010). Interplay of RNA Elements in the Dengue Virus 5′ and 3′ Ends Required for Viral RNA Replication ▿ . *J Virol, 84*(12), 6103-6118.

Gebhard, L. G., Filomatori, C. V., & Gamarnik, A. V. (2011). Functional RNA Elements in the Dengue Virus Genome. *Viruses, 3*(9), 1739-1756.

Guzman, M. G., Halstead, S. B., Artsob, H., Buchy, P., Farrar, J., Gubler, D. J., Peeling, R. W. (2010). Dengue: a continuing global threat. *Nat Rev Microbiol, 8*(12 Suppl), S7-16.

Hussain, M., & Asgari, S. (2014). MicroRNA-like viral small RNA from Dengue virus 2 autoregulates its replication in mosquito cells. *111*(7), 2746-2751.

Iglesias, N. G., & Gamarnik, A. V. (2011). Dynamic RNA structures in the dengue virus genome. *RNA Biology, 8*(2), 249-257.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol, 33*(7), 1870-1874.

Leitmeyer, K., Vaughn, D., Watts, D., Salas, R., Villalobos, I., de, C., Rico-Hesse, R. (1999). Dengue virus structural differences that correlate with pathogenesis. *J Virol, 73*(6), 4738-4747.

Paranjape, S. M., & Harris, E. (2010). Control of dengue virus translation and replication. *Curr Top Microbiol Immunol, 338*, 15-34.

Rico-Hesse, R., Harrison, L. M., Salas, R. A., Tovar, D., Nisalak, A., Ramos, C., da Rosa, A. T. (1997). Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. *Virology, 230*(2), 244-251.

Roche, C., Cassar, O., Laille, M., & Murgue, B. (2007). Dengue-3 virus genomic differences that correlate with in vitro phenotype on a human cell line but not with disease severity. *Microbes Infect, 9*(1), 63-69.

Rodriguez-Roche, R., & Gould, E. A. (2013). Understanding the dengue viruses and progress towards their control. *Biomed Res Int, 2013*, 690835.

Salgado, D. M. P., César A Vega, Martha Rocío Garzón, Marisol Castro, Dolly Rodríguez, Jairo Antonio. (2008). Dengue hemorrhagic fever mortality in children: beyond shock. *Infect., 12*(1), 21-27.

Selisko, B., Wang, C., Harris, E., & Canard, B. (2014). Regulation of Flavivirus RNA synthesis and replication. *Curr Opin Virol, 0*, 74-83.

Twiddy, S. S., Farrar, J. J., Vinh Chau, N., Wills, B., Gould, E. A., Gritsun, T., Holmes, E. C. (2002). Phylogenetic relationships and differential selection pressures among genotypes of dengue-2 virus. *Virology, 298*(1), 63-72.

Usme, J., Gómez, A., & Gallego, J. (2012). Molecular detection and typing of dengue virus by RT-PCR and nested PCR using degenerated oligonucleotides. *Salud, Barranquilla, 28*(1), 1-15.

Vaughn, D. W., Green, S., Kalayanarooj, S., Innis, B. L., Nimmannitya, S., Suntayakorn, S., Nisalak, A. (2000). Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *J Infect Dis, 181*(1), 2-9.

Velandia, M. L., & Castellanos, J. E. (2011). Dengue virus: structure and viral cycle. *Infect., 15*(1), 33-43.

WHO. (2009). DENGUE GUIDELINES FOR DIAGNOSIS, TREATMENT, PREVENTION AND CONTROL. In W. H. Organization (Ed.), (pp. 1-160). Switzerland: Geneva.

You, S., & Padmanabhan, R. (1999). A novel in vitro replication system for Dengue virus. Initiation of RNA synthesis at the 3'-end of exogenous viral RNA templates requires 5'- and 3'-terminal complementary sequence motifs of the viral RNA. *J Biol Chem, 274*(47), 33714-33722.

Yu, L., Nomaguchi, M., Padmanabhan, R., & Markoff, L. (2008). Specific requirements for elements of the 5' and 3' terminal regions in flavivirus RNA synthesis and viral replication. *Virology, 374*(1), 170-185.

Zhou, Y., Mammen, M. P., Jr., Klungthong, C., Chinnawirotpisan, P., Vaughn, D. W., Nimmannitya, S., Zhang, C. (2006). Comparative analysis reveals no consistent association between the secondary structure of the 3'-untranslated region of dengue viruses and disease syndrome. *J Gen Virol, 87*(Pt 9), 2595-2603.